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Phylogeography and Species Status of Ramphogordius sanguineus

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PHYLOGEOGRAPHY AND SPECIES STATUS OF

RAMPHOGORDIUS SANGUINEUS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

PHYLOGEOGRAPHY AND SPECIES STATUS OF *RAMPHOGORDIUS SANGUINEUS*

By Cora Ruth Runnels, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

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Ramphogordius sanguineus (Rathke 1799) is a gregarious nemertean with a worldwide distribution and found mainly on hard substrates associated with mussels, oysters and other organisms of the fouling community. Asexual reproduction occurs by spontaneous fragmentation and only anecdotal accounts of sexual reproduction exist. This is the first phylogeographic study of *R. sanguineus* as well as the first species delimitation analyses employing DNA markers. Analysis of the mitochondrial gene *nad6* and nuclear ISSR markers showed little diversity among geographically widespread populations, but AMOVA analyses of both markers revealed moderate to high genetic differentiation. Populations from Maine and Massachusetts exhibited the highest level of differentiation. These findings are consistent with predictions for invertebrates lacking a planktonic larval stage. Results of the *nad6* tree-based delimitation analysis were in agreement with modern morphological and histocompatibility observations, suggesting that *R. sanguineus* is a single species and that a former division into four separate species was solely based on geographic location.

Introduction

The phylum Nemertea contains approximately 1,275 species (Kajihara *et al*. 2008) occupying marine, freshwater and terrestrial habitats worldwide (Gibson 1995). These unsegmented worms are characterized by an eversible proboscis that is used primarily for capturing prey. As predators, nemerteans play an important role in marine communities and are potentially capable of altering the population structure of prey species (Thiel and Kruse 2001; Caplins *et al*. 2012). Some species have significant economic impacts on commercial crab and soft-shell clam fisheries (Wickham *et al.* 1984; Shields and Kuris 1988; Kuris 1993; Bourque *et al.* 1999). They are proving to be important bio-indicator species as well (Thiel and Zubillaga 1998) and are useful in understanding the influences of external stimuli on reproductive cycles (McEvoy *et al.* 1998).

Although significant in a wide-range of research, the phylum Nemertea is considerably understudied compared to most animal phyla. Few investigations have focused on relationships below the family level, and much of the current nemertean taxonomy is based on a limited number of morphological characters (Gibson 1985; Moore and Gibson 1993; Sundberg 1993; Sundberg and Svensson 1994). Because intraspecific variation and interspecific similarities are common within the phylum, it is likely that many taxa are currently recognized as either complexes of several species or are synonymous with others (Gibson 1985; Sundberg 1993; Moore and Gibson 1993; Sundberg and Svensson 1994; Gibson 1995). For example, the allozyme analysis of Sundberg and Janson (1988) on three morphologically distinct populations of *Oerstedia*

dorsalis revealed two populations as almost genetically identical but the third as a separate species. Additionally, a histological comparison of "brown" and "cherry" colored *Lineus torquatus* collected from the Sea of Japan did not reveal morph-specific differences, while enzyme electrophoresis analysis determined the two as genetically distinct species (Manchenko and Kulikova 1996). Strand and Sundberg (2005a) analyzed the intrageneric variation in *Tetrastemma* species using a species delimitation method that included assessment of the genetic variation and phylogenetic relationships between morphologically distinct forms with the mitochondrial cytochrome oxidase-1 (*cox1*) gene. They found little concordance between morphological species and evolutionary lineages (Strand and Sundberg 2005a). Strand and Sundberg (2005b) further determined, using tree-based species delimitation in which the phylogeny was based on partial 18S ribosomal RNA (18S rRNA) sequences using Bayesian and maximum likelihood analyses, that although *Tetrastemma* is a monophyletic group not all the species assigned to this group should be placed in this genus.

Even less attention has been concentrated on population level analyses of nemerteans and existing investigations are limited to only a few species. For example, Rogers *et al*. (1998a) determined that Antarctic heteronemertean *Parborlasia corrugatus* populations exhibited lower heterozygosity and genetic variation than other species with similar larval stages. Based on the genetic differences between Antarctic and South American populations of *P. corrugatus*, Thornhill, *et al.* (2008) revealed high differentiation between these populations and suggested they represent two cryptic species. Furthermore, Rogers *et al.* (1998b) concluded that North American and

European populations of the heteronemerteans *Lineus viridis* and *Lineus ruber* exhibited a moderate degree of population structure with little evidence of genetic differentiation between populations separated by large geographic distances. Similarly, the hoplonemertean *Malacobdella arrokeana* lacks differentiation over significant geographical distances (Alfaya *et al*. 2012).

Ramphogordius sanguineus (Rathke 1799) is a gregarious heteronemertean found beneath stones and among mussels and oysters in temperate intertidal zones of the Atlantic and Pacific coasts of North America, Europe and Asia (Coe 1943; Riser 1994). This species ranges in length from 0.5 cm to 20 cm and from 1 to 3 mm in diameter (Coe 1943; Riser 1994). They possess 2-8 pairs of ocelli, long cephalic grooves along each side of the head and have a tendency to contract into a spiral coil when disturbed (Coe 1943; Riser 1994). Smaller individuals tend to be whitish-grey; whereas larger individuals are often found in varying colors such as olive, red, brown or green (Coe 1943; Riser 1994). Riser (1994) suggested that diet might affect color variation.

Asexual reproduction occurs in this species by spontaneous fragmentation with regeneration of fragments containing a portion of the lateral nerve cords (Coe 1930, 1931, 1943). Encystment of fragments often occurs, which may allow for protection against predation and parasites during the regenerative process (Coe 1930, 1943). Although there have been limited observations of oocyte formation and embryonic development, it is unclear whether sexual reproduction regularly occurs (Coe 1931, 1943; Riser 1974). Coe (1899) reported that in captivity eggs developed to the gastrulae but provided no further information on embryonic development. Periods of normal oocyte

development have been reported, although observations suggest it to be abortive (Coe 1943; Gontcharoff 1951; Cantell 1989; Riser 1994). A larval dispersal stage is thus unknown (Riser 1993, 1994). *R. sanguineus'* closest relatives possess a benthic larva that develops into a juvenile within egg capsules attached to the substrate. Both *R. sanguineus* adults and cysts could easily be transported on ships and floating debris accounting for its global distribution (Riser 1993, 1994).

Despite the abundance of *R. sanguineus* along the coastlines of primarily temperate regions of the world, it is greatly understudied. Although it has been included in phylogenetic analyses of nemerteans (Sundberg and Saur 1998; Thollesson and Norenburg 2003) and in Lineid species boundary studies (Williams *et al.* 1983; Rogers *et al.* 1993, 1995; Tarpin and Bierne 1998), no population-level investigations of this widespread species exist. Invertebrate species with long-lived planktonic larvae (with exceptions see Goetze 2011) are expected to exhibit little differentiation over large distances because of larval dispersal and those lacking such larvae are predicted to show increased differentiation (see Ward 1989; Palumbi 1992; Avise 2000). *R. sanguineus* which likely lacks a planktonic larval stage, therefore presents an ideal organism to further test the latter prediction.

A number of studies have focused on the species status of *R. sanguineus.* Bierne *et al.* (1993) showed little or no difference between histocompatibility characters, and any differences were attributed to intraspecific variation. They, therefore, suggested that *Lineus sanguineus, L. nigricans, L. vegetus* and *L. pseudolacteus* be assigned to a single species: *L. sanguineus* with three subspecies *L.s. sanguineus, L.s. nigricans* and *L.s.*

pseudolacteus without presenting explicit criteria for such a division. These results further suggested that original species designations were based solely on geographic location. Riser (1993) partial agreed, but argued *L. psuedolacteus* should be a separate species because of morphological differences such as color pattern and internal anatomy of preserved specimens (Bierne *et al.* 1993). Riser (1994) erected the genus *Myoisophagus* that comprised *Lineus lacteus, L. psuedoacteus,* and *L. sangiuneus. Myoisophagus* was later determined to be invalid, and these species were reassigned to the genus *Ramphogordius* (Riser 1998). Species delimitation analyses employing DNA markers are lacking.

Due to its high mutation rate and nonrecombining mode of transmission, mitochondrial DNA (mtDNA) is a useful marker for phylogeographic studies to estimate relatedness within and among populations (Avise *et al.* 1979, 1987). However, because of introgression, selection and incomplete lineage sorting of mtDNA between populations, mitochondrial markers may not give a true representation of the history of the populations (Avise 1994; Ballard *et al.* 2004). Therefore, to ensure a more accurate representation of the population structure, nuclear markers are used conjunction with mtDNA (see Duran *et al*. 2004b, 2004c). Nuclear inter-simple sequence repeats (ISSRs) are hypervariable, dominant markers that quickly and reliably examine gene flow among populations, genetic diversity and species boundaries (Wolfe 2005). Although originally designed for plants (Wolfe 2005), their usefulness in examining genetic variation in animals has been effectively demonstrated (e.g., Reddy *et al.* 1999; Abbot 2001).

Asexually reproducing organisms, such as *R. sanguineus,* typically demonstrate low genetic diversity (Burger 1999), but ISSRs are sensitive enough to distinguish between closely related individuals and are informative in population studies of animals with low levels of within-population variation (Zietkiewicz *et al.* 1994; Reddy *et al.* 1999; Abbot 2001; Wolfe 2005). To obtain a robust assessment of variation among populations of *R. sanguineus,* ISSRs were employed in conjunction with the mitochondrial NADH dehydrogenase subunit 6 (*nad6*) gene in this study.

The current study evaluated population structure and genetic diversity of *R. sanguineus* over most of its known geographic range using mitochondrial and nuclear markers and tested with molecular markers, whether *R. sanguineus* represents a single species using a tree-based delimitation method. These results will provide insight into the genetic diversity and population structure of this widespread worm allowing a test of the predictions for marine invertebrates lacking planktonic larvae (see above). The data also will be the first molecular test of whether *R. sanguineus* represents a single species or a complex of species, as originally inferred from geographical distribution.

Materials and Methods

Sample Collection and DNA Extraction

Individuals from the West coast of North America, Europe and Asia were obtained from Jon L. Norenburg, Smithsonian Institution. Worms were collected by Cora R. Runnels and James M. Turbeville from the East coast of North America (Figure 1).

Former species designation, population number, location, population, sample size, latitude and longitude are listed in Table 1.

Samples were stored at -20°C in either RNA later (Ambion, Inc) or 95% ethanol prior to processing. DNA was extracted from approximately 25 mg of tissue for each individual using the QIAamp DNA Mini Kit (Qiagen, Inc) following the standard protocol for tissues.

Amplification and Sequencing of the Mitochondrial nad6 Gene

The *nad6* gene was selected for this study, because, unlike standard mitochondrial markers such as *cox1* and the large subunit ribosomal RNA (*rrnL*), it exhibited variation among test populations. The entire *nad6* gene (522 base pairs) was amplified by polymerase chain reaction (PCR) using the following primer sets: foward 5'-GATTTTTCCTCTTTCGATGTCAAG-3' or 5'CTCTTTCGATGTCAAGA AATGG-3' and reverse 5'-ATTAACAACCTTCAAAACAGGATG-3'. The PCR cycle profile was as follows: initial denaturation at 94°C for 1 minute, followed by 40 cycles of 94°C denaturation for 10 seconds, annealing between 45°C and 53°C for 25 seconds, and extension at 68°C for 40 seconds with a final extension at 68°C for 4 minutes. The PCR product was visualized by agarose gel electrophoresis with ethidium bromide staining prior to purification and sequencing.

PCR reactions were purified with ExoSAP-IT (USB, Inc.) and sequenced using the Dynamic ET Dye Terminator Cycle-Sequencing (Amersham Biosciences) kit. Sequencing reactions were purified using Montage MultiScreen SEQ sequencing reaction

cleanup plates (Millipore Corp.). Sequence data were obtained using an Amersham Biosciences Megabace 1000 automated DNA sequencer. The *nad6* gene was sequenced in both directions for each individual, and the fragments were assembled using AlignIR (LiCor, Inc.) with any sequencing errors corrected by strand comparison. The refined sequences were then aligned using ClustalX (Ramu *et al.* 2003). *Nad6* nucleotide sequences and translated amino acid sequences were entered into NCBI BLAST (Altschul *et al.* 1990), and both returned strong matches with *nad6* from published mitochondrial genomes of other nemerteans. The BLAST results were taken as confirmation that the correct product had been amplified and sequenced. AlignIR indicated double peaks at 3 positions in all 6 individuals from the France. One peak was a C (cytosine), and the other was a T (thymine). This was taken as evidence of heteroplasmy, and because pyrimidines are involved, the ambiguity symbol "Y" was used at the appropriate positions.

ISSR Amplification and Scoring

ISSRs were amplified by single-primer PCR using the primers 810 $(GA)_8T$, 826 $(AC)_8C$ and 842 $(GA)_8YG$ (UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia) at annealing temperatures of 45°C, 52°C and 48°C respectively. The PCR profile was as follows: initial denaturation at 94°C for 1 minute 30 seconds, followed by 35 cycles of 94°C denaturation for 40 seconds, annealing for 45 seconds, and extension at 72°C for 1 minute 30 seconds followed by a final extension at 72°C for 5 minutes. ISSR bands were visualized on a 1.5% agarose gel run at 4-5V/cm for 180

minutes and then stained with ethidium bromide. ISSR bands were scored as present (1) or absent (0) for all individuals. Faint and non-reproducible bands were excluded. Amplifications were repeated for some individuals to test for the reproducibility of the bands.

Nad6 Data Analysis

Gene trees were reconstructed from aligned *nad6* sequence data with maximum parsimony (MP) and maximum likelihood (ML) analysis to estimate relationships among haplotypes. MP analysis was performed in PAUP* 4.0 (Swofford 2002) using a heuristic search with 100 random addition replicates and TBR branch swapping. A ML tree was constructed using RAxML as implemented on the Cipres Science Gateway (http://www.phylo.org/sub_sections/portal/) with bootstrap values. The resulting tree was analyzed and edited in Dendroscope (Huson *et al.* 2007). The closely related nemertean *Ramphogordius lacteus* was used as the outgroup.

Haplotype diversity was calculated by hand using the standard formula (Nei and Tajimia 1981) and nucleotide diversity was calculated using DnaSP v. 5.10.1 (Rozas 2009). A haplotype network was constructed to describe relationships between *nad6* haplotypes within *R. sanguineus* populations using statistical parsimony as implemented in TCS, since gene trees could be nonhierarchical or contain extant ancestral nodes (Posada and Crandall 2001). A 95% statistical parsimony network (Templeton *et al.* 1992) was constructed using the software TCS (Clement *et al.* 2000). Analysis of Molecular Variance (AMOVA) was utilized to examine genetic variation within and

among populations (Excoffier *et al*. 1992). The analyses were performed with the GeneticStudio software package (Dyer 2006), using 999 permutations to test the significance and were analyzed as haplotype markers.

The Mantel test analyzes the correlation between two matrices (Mantel 1967). The Mantel test was performed using Manteller (Dyer 2006) to determine the correlation between genetic distances and geographic distances of populations. The geographic distance matrices were constructed from the latitudinal and longitudinal coordinates of the populations using the software ArcMap (http://support.esri.com/datamodels), and the *nad6* genetic distance matrices were constructed from AMOVA distances generated in GeneticStudio (Dyer 2006).

ISSR Data Analysis

The following ISSR descriptive statistics were calculated: total number of bands scored, size range of bands for each primer, average number of bands per primer for each region and for total, percentage of polymorphic loci per primer for each region and for total, average number of bands for all primers for each region and for total, average number of bands per primer for each region and for total, and percentage of polymorphic bands for all primers per region.

ISSR amplification products were scored as band presences or absences [1 (present) or 0 (absent)]. Similarity index matrices based on the number of shared fragments were generated with program package TREECON (Van de Peer & De Wachter 1994). The Nei and Li coefficient ($\hat{S}$), (Nei and Li 1979, equation # 10), which is

equivalent to the Dice- Sørensen distance (Dice 1945; Sørensen 1948; see Archibald *et al.* 2006) was used for distance matrix computation, as it excludes shared absences whose homology cannot be evaluated. A;/ tree was reconstructed from the distance values using Neighbor Joining (NJ) as implemented in TREECON (Van de Peer & De Wachter 1994).

AMOVA was accomplished as above and analyzed as dominant markers. The AMOVA distance option was used in the GeneticStudio software package (Dyer 2006) when analyzing ISSR data. Using a Euclidean metric is undesirable for ISSRs, because it will count shared band absences as positive matches, when in reality shared absences are unlikely to be homologous (Wolfe *et al.* 1998). Although AMOVA was originally designed to work with a Euclidian distance metric, although non-Euclidian metrics are also acceptable (Excoffier *et al.* 1992). The Mantel test was performed as above. ISSR genetic distance matrices were constructed in GeneticStudio (Dyer 2006).

Species Delimitation

A tree based exclusivity approach for delimiting species was implemented to test the species status of *R. sanguineus* (reviewed in Sites and Marshall 2003). Individuals of *R. sanguineus* from populations previously designated *L. vegetus, L. sanguineus, L. pseudolacteus, L. socialis,* and *L. nigricans* served as focal species (Table 1). The analysis of higher-level nemertean relationships by Thollesson and Norenburg (2003) and analysis of (Sundberg and Saur 1998) strongly suggest that *L. viridis, L. ruber* and *R. lacteus* are appropriate candidates. Therefore, these three species were included in the analysis. Haplotype trees were estimated with parsimony from *nad6* sequence data and

correlated with haplotype localities to infer species boundaries. Identical sequences for each geographic location were excluded from the data set to make the analyses tractable.

For parsimony analysis, a heuristic search was employed with 1000 random addition replicates. Bootstrap analyses were conducted (1000 replicates) using a full heuristic search (10 random addition replicates). A Bayesian tree was inferred using MrBayes as implemented on the Cipres. Two independent Markov Chain Monte Carlo chains were run for 10^6 generations, employing the HKY+G model as inferred by JModelTest (Posada 2008) with gamma distributed rates; Gamma distribution was approximated with 4 categories. Trees were sampled every 1000 generations. Burn in fraction was set to 25%. Posterior probabilities were used as a measure of clade support. Results of the tree-based analysis were then compared with morphological and histocompability analyses of Riser (1993, 1994) and Bierne *et al.* (1993) that originally described *R. sanguineus* as a single species.

Results

Nad6 Data Analysis

Sequence data were obtained for 522 bp of *nad6* from a total of 324 *R. sanguineus* individuals representing 29 geographic locations and 5 former species designations (Table 1). Because of minimal variation among individuals (uncorrected distances 0% - 1.52%, 14 parsimony informative sites), no meaningful resolution was obtained using ML or MP analyses; therefore the results are not shown. Haplotype diversity was

moderately high (H = 0.77; SD = 0.0156), but nucleotide diversity was very low (π = 0.00152; SD = 0.00015).

A haplotype network constructed using statistical parsimony (95% connection limit) revealed twelve haplotypes. The inferred ancestral haplotype was found in seven populations: 4 individuals from California, 2 individuals from Washington, United Kingdom, New Jersey and New Hampshire respectively and 1 individual from Maine. The ancestral haplotype being defined as the haplotype that is most frequent and has the most low frequency haplotypes connected to it (see Crandall and Templeton 1993). Two additional widespread haplotypes originated directly (one step) from the ancestor with all others exhibiting a more restricted geographic distribution. One haplotype was found only in Maine and Massachusetts populations and 4 unique haplotypes were found in each of 4 United Kingdom individuals (Figure 2). No absolute geographic division of populations was inferred. Divergence from the inferred ancestral haplotype ranged from 1 to 6 steps.

Pairwise Φ_{st} values were obtained for *nad6* sequence data in from geographical location by combining sample locations into regions. Sample locations that were small such as New York (n = 2) were combined with populations within the same regions (Tables 2). The north region individuals showed high level of differentiation from all other populations including those from the other two North American Atlantic coast regions (Φ_{st} = 0.5642 to 0.8002). A lower level of differentiation is inferred between the South and Rhode Island (Φ_{st} = 0.1062). Rhode Island also exhibited a lower Φ_{st} value (0.2909) with West than with South (Φ_{st} = 0.5623). This suggested there is more gene

flow or shared evolutionary history between populations between Rhode Island and West than between South and West. There was moderate differentiation between Rhode Island and Europe (Φ_{st} = 0.2146). The results also indicated moderate differentiation between the West region and both Europe (Φ_{st} = 0.1532) and Japan (Φ_{st} = 0.1435). Japan was highly differentiated from all regions (Φ_{st} = 0.2532 to 0.8002) except for West (Φ_{st} = 0.1435). The China region exhibited the highest differentiation from all other populations, with somewhat lower Φ_{st} values between China and Rhode Island (Φ_{st} = 0.2856) and China and Europe (Φ_{st} = 0.1998) (Table 2).

Mantel tests were performed to determine the correlation between genetic distance and the geographical distance between the sampling sites of *R. sanguineus*. There was a significant relationship between physical distance and genetic distance for *nad6* data ($p < 0.0001$), therefore suggesting isolation by distance.

ISSR Data Analysis

A total of 304 individuals were included in the ISSR analyses. Bands for individuals from two Rhode Island, two California, all (4) Spain, one Japan and all (11) China populations either did not amplify or were faint and therefore were not included in the ISSR analyses. The primer numbers, primer sequence, size range of bands for each primer, total number of bands scored for each primer and total number of genetic loci scored are listed in Table 4. The total number of bands scored with primer 810 was 5, primer 826 was 10 and 842 was 13. The total number of bands scored across all primers was 28. The size range of bands across all primers was between 325 kb and 3200 kb. The

size range of the bands for primer 810 is between 1900 kb and 3100 kb, primer 826 is between 700 kb and 3000 kb, and primer 842 is between 325 kb and 3200 kb (Table 4).

ISSR statistics were calculated based on regions, which were determined based on AMOVA results (Table 3) and small sample sizes. The regions were combined as follows: North contained 75 individuals from Maine (N=58), Massachusetts (N=14) and New Hampshire (N=3), Rhode Island contains 67 individuals from Rhode Island (N=62), New Jersey (N=3) and New York (N=2), South contains 85 individuals from South Carolina (N=81), North Carolina (N=1) and Florida (N=3), West contains 23 individuals from California (N=15) and Washington (N=8), Europe contains 37 individuals from United Kingdom (N=31) and France (N=6), Japan contains 17 individuals from Japan. The average number of bands scored and % polymorphic band for each primer and region are given in table 5.

A tree was constructed from ISSR data employing Nei-Li distances (Figure 3) and NJ. Distances ranged from 0.00% to 73.91%. There was only partial resolution of geographic populations. For example, there were several groups of North population individuals throughout the tree. This was the case for other populations as well (Figure 3).

Pairwise Φ_{st} values were obtained for ISSR bands and combined with populations within the same regions (Tables 3). Bands for individuals from two Rhode Island, one South Carolina, two California, all (4) Spain, one Japan and all (11) China populations either did not amplify or were faint and therefore were not included in the AMOVA ISSR analyses. Population AMOVA values based on nuclear ISSR data are mostly congruent

with those for the *nad6* gene (Table 3). North individuals showed a high level of differentiation from all other populations (Φ_{st} =0.4338 to 0.7612). Lower levels of differentiation were inferred between the South and Rhode Island (Φ_{st} = 0.2096), Rhode Island and Europe (Φ_{st} = 0.1321) and West and Japan (Φ_{st} = 0.1995). Results from the two markers conflicted only between Europe and South, with Φ_{st} values for *nad6* (0.2146) lower than for ISSR (0.4243) data (Tables 2 and 3). However, both values indicate moderate to high differentiation.

Mantel tests were performed using ISSR genetic distance matrices constructed from AMOVA distances and the geographic distance matrices run as above. There was a significant relationship between physical distance and genetic distance for both the ISSR data ($p < 0.0001$), therefore suggesting isolation by distance.

Species Delimitation

Bierne *et al.* (1993) suggested *R. sanguineus* was previously diagnosed based on geographic location with *L. socialis* from Atlantic North American coasts, *L. vegetus* from Pacific North American coasts, *L. sanguineus* from Europe Atlantic coast, *L. pseudolacteus* from shores of the English Channel and *L. nigricans* from the Mediterranean coast of France. MP and Bayesian analysis provide no support for these designations (Figures 4 & 5). In the MP analysis all *R. sanguineus* individuals formed a single clade with high bootstrap values (100%) and none of the individuals previously designated as separate species based on geographic location formed separate clades.

Results from the Bayesian analysis were similar providing no support for separate species based on geographic location.

Discussion

Genetic Variation

The mitochondrial gene *nad6* exhibited low sequence variability in this study of *R. sanguineus* with only 14 variable positions from the 522 base pairs sequenced among 324 individuals. Nucleotide diversity ($\pi = 0.00152$) was low for a globally distributed species, although similar results were found for other invertebrates (see below). Twelve haplotypes were determined from the *nad6* data for the entire sample (324 individuals). Haplotype diversity for the entire population was high ($H = 0.77$).

Phylogenetic analyses for *nad6* showed no resolution because of the limited number of variable informative sites. For similar reasons, the ISSR tree inferred from Nei-Li distances recovered only partial resolution of some geographical populations with weak support (Figure 3). However, AMOVA analyses of both markers revealed differentiation between geographic populations.

The correlation of low nucleotide diversity with genetic differentiation among populations is not unique to the nemertean. For example, partial mitochondrial *cox1* gene analysis revealed low sequence divergence, but differentiation between eight populations over a wide geographical area in the marine sponge *Crambe crambe* (Duran *et al.* 2004a). Nucleotide diversity ($\pi = 0.0006$) was lower than that for *R. sanguineus* ($\pi = 0.00152$).

Similarly, genetic structure and low gene flow between close populations were inferred and only two haplotypes were found in 6 locations (Duran *et al.* 2004a). However, the different haplotype frequencies found among populations revealed genetic structure and low gene flow between close populations as expected from the dispersal biology of the species (Duran *et al.* 2004a). Larvae are short-lived (24-72 h; Duran *et al.* 2004a), and sponge fragments capable of regenerating a new individual can be transported short distances. Both could function in short range dispersal among connected bodies of water, but neither possesses the structural or physiological mechanisms necessary for movement across significant distance (see Reiswig & Frost 2010).

Inference of differentiation among geographically distant populations of *R. sanguineus* contrasts with results for other nemerteans. Rogers *et al.* (1998b) analyzed allozyme data from two heteronemerteans (*L. ruber* and *L. viridis*) that are also common inhabitants of the fouling community. The results suggest a lack of differentiation among populations sampled in the UK, France and North America, which may be due in part to introductions by rafting or from boats and possibly passive dispersal. These worms lack a planktonic larva, but the juveniles of *L. viridis* could potentially be transported in the water column (Rogers *et al.* 1998b). Balancing selection on the loci investigated was also invoked as a reason for genetic homogeneity. Analyses of additional mitochondrial and nuclear markers will be necessary to further clarify the genetic structure in these worms.

Thornhill *et al.* (2008) analyzed *rrnL* and *cox1* sequence data in the heteronemertean *P. corrugatus* and similarly found little genetic diversity over a large geographical scale. Overall nucleotide diversity was relatively low (0.01566), although it

was higher than that for *R. sanguineus*. Thirty-two separate *cox1/rrnL* haplotypes were detected in the 86 samples. The *rrnL* fragment was less variable and less informative with 9 unique *rrnL* sequences, whereas there were 25 unique *cox1* sequences. Two haplotypes accounted for the majority of the samples (51 of the 86 individuals). Haplotype diversity was high (0.82). A larval dispersal phase is known for *P. corrugatus* and Antarctic and sub-Antarctic populations separated by large distances were found to be relatively homogenous. Gene flow was restricted only between South American populations and the former populations by the Antarctic Polar Front, which is recognized as a strong barrier to dispersal (see Thornhill *et al.* 2008).

The entocommensal nemertean *M. arrokeana* found in clams along the Patagonian Gulf using mitochondrial *cox1, rrnL* and nuclear ITS-2 markers revealed a lack of differentiation among populations separated by hundreds of kilometers (Alfaya *et al.* 2012). This species possesses a lecithotrophic larval phase in its life history suggesting limited dispersal capability, although larval residence time in the water column is unknown. As in *R. sanguineus*, nucleotide diversity was low (π = 0.001 - 0.002), but haplotype diversity high (H = 0.939). However, no differentiation was detected between four populations. This was attributed to the absence or "porousness" of barriers and intermittent changes in habitat (Alfaya *et al.* 2012). In the meiofaunal hoplonemertean *Ototyphlonemertes evelinae* and *O. erneba* gene flow due to larval dispersal has been inferred for populations that are separated by large distances. For other species of this genus, significant structuring is observed (Andrade *et al*. 2011).

Explanation of Pattern

The observed structure in populations of *R. sanguineus* can be explained in part by isolation by distance (IBD). The Mantel test showed a significant relationship between genetic distance and geographic distance for both ISSR data ($p < 0.0001$) and *nad6* ($p < 0.0001$) and therefore significant evidence of IBD.

The *nad6* and ISSR AMOVA results indicated high differentiation between the regions North and Rhode Island, South, West, Europe, Japan and China (Table 2). There is also structuring between Japan and Rhode Island and South as well as between China and South and West (Table 2). The ISSR AMOVA results showed high differentiation between North and Rhode Island, South, West, Europe and Japan (Table 3). This high level of differentiation could be explained by an event involving a very small number of individuals coupled with asexual reproduction, which is common in the species. A hydrological barrier, namely the Labrador Current, may be limiting dispersal between the North (ME, MA) and Rhode Island (RI, NY, NJ) populations. There was also structuring between Japan and Rhode Island, South and Europe as well as between South and West and Europe (Table 3).

A larval dispersal stage for this species is unknown and unlikely, given that its nearest relatives have larvae that are passed in capsules attached to the substrate (see Riser 1994 and phylogeny in Andrade *et al.,* 2011). It does produce "cysts" asexually, which are regenerating fragments enclosed in mucous that could potentially disperse over short distances, if they become dislodged from the substrate and entrained in the water current. Furthermore, this species is primarily a member of the fouling community of

hard-substrates, and the lack of appropriate substrates forming a global continuum might be expected to limit its distribution. These life history attributes are consistent with the IBD effect.

However, moderate differentiation was inferred using AMOVA between some populations separated by long geographic distances (Rhode Island and South, Rhode Island and Europe, West and Europe) suggesting some gene flow or common ancestry. Although, as mentioned previously, *R. sanguineus* most likely lacks a larval dispersal phase, its habitat preference makes it an ideal candidate for long-distance dispersal either encysted juveniles or mature individuals by rafting with debris in ocean currents or on boat hulls, which often are covered with fouling organisms (Coe 1930, 1943; Riser 1993, 1994).

Platyhelminthes, nematodes, annelids and some nemerteans are frequently found on floating macroalgae and other rafting fauna (reviewed in Thiel and Gutow 2005). Highsmith (1985) determined a potential for small individuals to disperse by rafting on macrophytes and seagrasses, which would provide shelter and food for small organisms for a considerable period of time. *R. sanguineus* could be transported among the byssal threads of mussels. Anthropogenic dispersal of these animals could have been occurring for centuries mediating gene flow between these populations. Rafting in major oceanic currents cannot be ruled out as a factor, but the frequency and speed of ship transport likely overshadows the contribution of rafting to the dispersal of this species greatly enhancing the probability of gene flow.

South Carolina and Rhode Island are near the Gulf Stream and both have high shipping traffic between these localities (http://www.nceas.ucsb.edu/globalmarine/impacts). The rocky substrate in South Carolina is recent (primarily 19th and 20th century structures) in comparison to the natural hard substrates found in the North East. Individuals may originally have been transported on ships from Rhode Island to South Carolina. This could explain the shared haplotypes and lower differentiation as compared with other populations. Such a dispersal mechanism could also explain the lower levels of differentiation between other populations such as Rhode Island and Europe and the West coast and Japan. It is important to stress, however, that verification of anthropogenic facilitation of dispersal through shipping will require careful quantification of the extent of shipping traffic between localities coupled with an assessment of ship fouling community biodiversity.

Species Delimitation

Bierne *et al.* (1993) suggested that species of fissiparous *Lineus* were previously diagnosed based on geographical location: *L. socialis* occurring on the Atlantic North American coasts, *L. vegetus* on Pacific North American coasts, *L. sanguineus* on the shores of European seas, *L. pseudolacteus* on the shores of the English channel and *L. nigricans* on the Mediterranean coast of France. These investigators carried out a histocompatibility analysis which revealed little or no difference between genetic characters and any differences were attributed to intraspecific variation and therefore

suggested that *L. sanguineus*, *L. nigricans*, *L. socialis*, *L. vegetus* and *L. pseudolacteus* be assigned to a single species, *L. sanguineus*.

Riser (1993) was in partial agreement but argued *L. psuedolacteus* be placed in a separate species because of morphological differences. Riser (1994) erected the genus *Myoisophagus* that comprised *L. lacteus, L. psuedolacteus* and *L. sanguineus*. *Myosophagus* was later determined to be invalid, and these species were reassigned to the genus *Ramphogordius* (Riser 1998).

The tree-based species delimitation analysis employed in this study resulted in all *R. sanguineus* individuals forming a clade with high bootstrap support (Figures 4 & 5). None of the individuals previously considered separate species formed subclades as would be predicted if they were distinct species. Thus, *nad6* sequence data analyses are congruent with morphological and histocompatibility characters suggesting that former species (*L. socialis, L. vegetus, L. sanguineus, L. pseudolacteus* and *L. nigricans*) should all be synonymized with *R. sanguineus*.

Conclusion & Future Directions

The results of genetic structure analyses reveal moderate to high differentiation among populations that are separated by large distances and data support IBD as a likely explanation of the exhibited pattern. However, a hydrological barrier is suspected for the high differentiation between the North (ME, MA) and the Rhode Island (RI, NJ, NY) populations. These worms likely lack a larval dispersal phase and occasional gene flow may be facilitated by anthropogenic transport or rafting. The results are consistent with

that expected for animals with short-lived larvae of minimal dispersal potential and those lacking larvae (see Palumbi 1992; Avise 2000) and extend our knowledge of nemertean phylogeography. Tree-based species delimitation with *nad6* sequence data also support synonymization of four former geographic species with *R. sanguineus*, illustrating the utility of DNA sequence data for nemertean taxonomy.

This study also provides a framework for expanded investigations and several recommendations are provided. Larger sample sizes, especially for New Hampshire, New Jersey, New York, Florida, Washington, California, Spain, France, Japan and China will allow a more accurate representation of the nucleotide, haplotype and ISSR diversity for *R. sanguineus* and also result in more comprehensive analyses of population structuring on a global scale. Furthermore, a more thorough assessment can be accomplished by developing microsatellite markers. Unlike ISSRs, which are dominant markers, co-dominant microsatellites would allow calculations of allele frequencies and could potentially provide evidence for clonal vs. sexual populations.

Although phylogenetic analysis of the *nad6* gene is congruent with morphology, supporting the hypothesis that *L. socialis, L. vegetus, L. sanguineus, L. pseudolacteus* and *L. nigricans* belong to the same species (*R. sanguineus*), species delimitation can be strengthened with the addition of nuclear markers. Potential markers include internal transcribed spacers of the ribosomal RNA repeat unit.

Figures

Figure 1. Collection Site Locations. *R. sanguineus* individuals from North America, Europe and Asia were obtained from Jon L. Norenburg, Smithsonian Institution Individuals from the East Coast of North America were collected by Cora R. Runnels and James M. Turbeville.

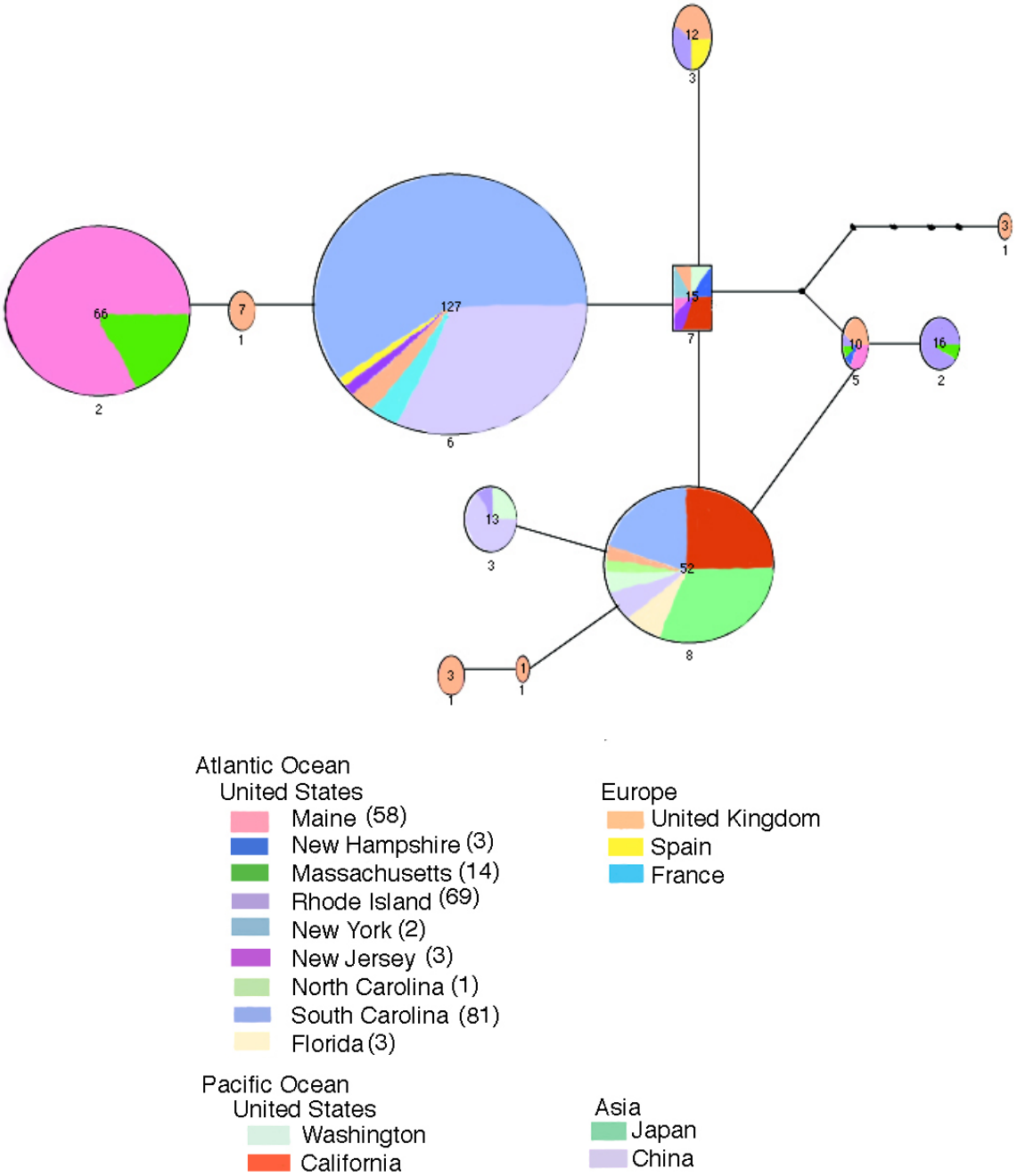

Figure 2. Haplotype Network. A 95% statistical parsimony haplotype network (Templeton et al. 1992) constructed using the software TCS (Clement et al. 2000) describing relationships between *nad6* haplotypes within *R. sanguineus* populations. Numbers in circle represent number of individuals located in each haplotype. The number of haplotypes within each circle is provided below.

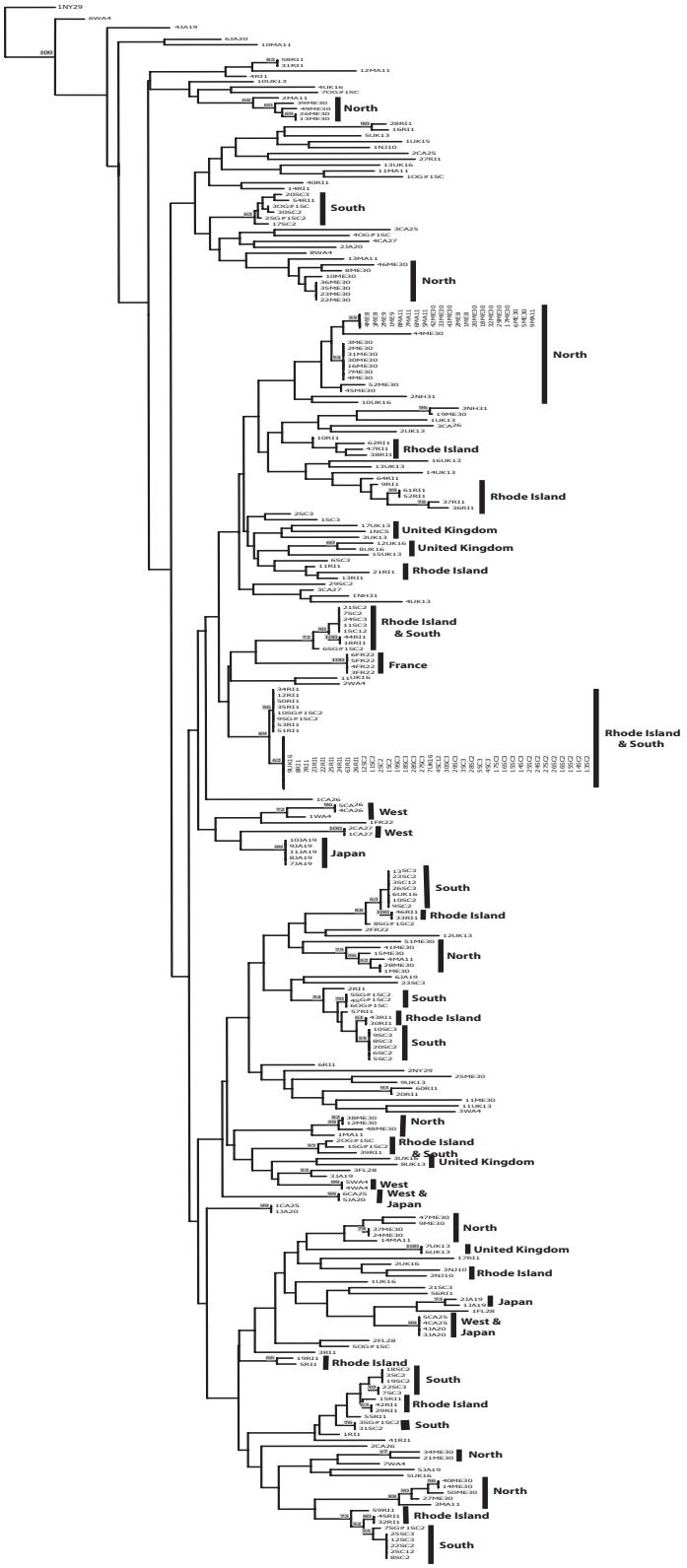

Figure 3. Neighbor Tree Reconstructed from ISSR Genotypes. Geographic populations were not fully resolved.

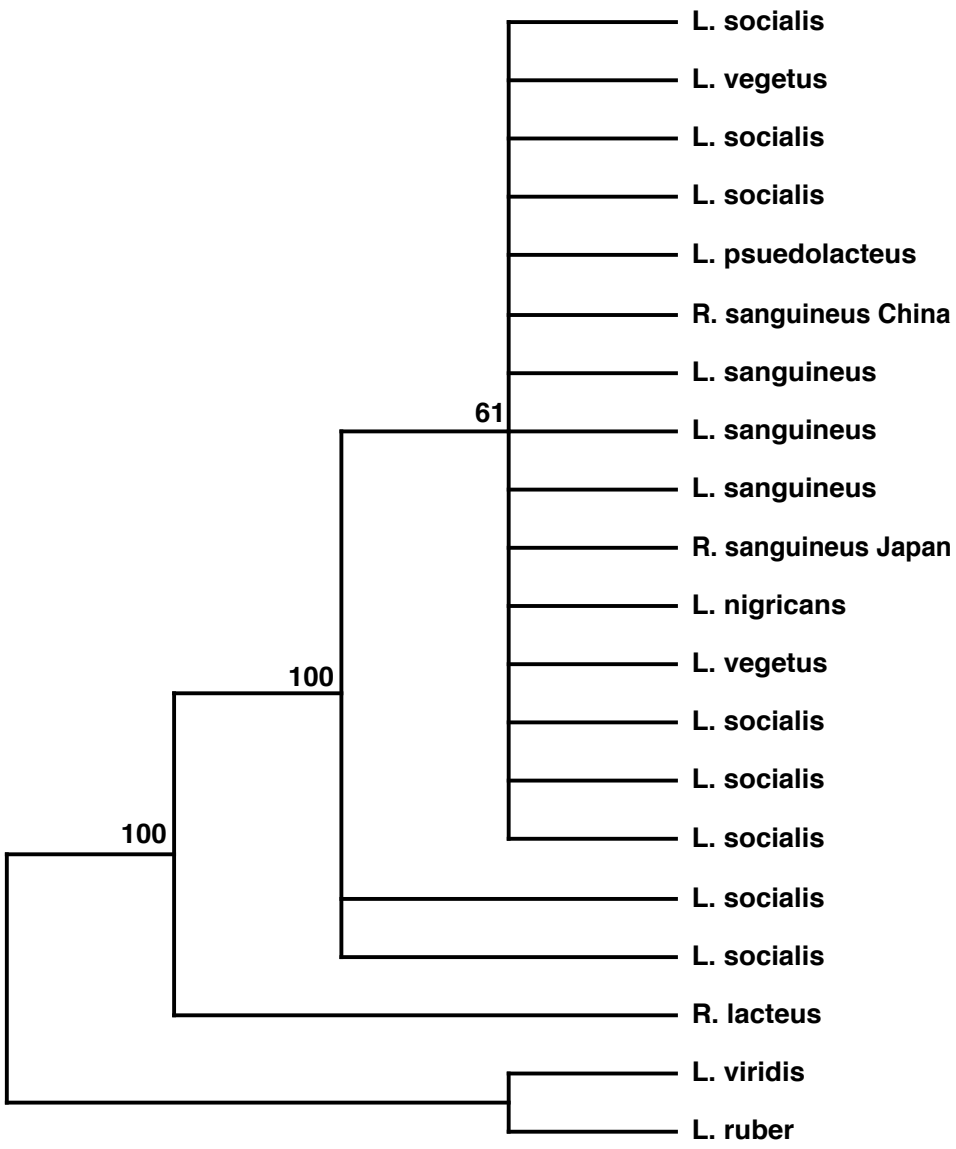

Figure 4. Maximum Parsimony Tree Based Delimitation Using *nad6* Sequence of Representatives of all "Geographic Species." Strict consensus of 240 equally parsimonious trees. Former designations of *R. sanguineus* are indicated as *L. socialis*, *L. psuedolacteus, L. sanguineus, L. vegetus* and *L. nigricans* and were originally defined on the basis of geographic location. These do not form distinct groups. The reference taxa are *L. ruber, L. viridis* and *L. lacteus.* Bootstrap percentages are at nodes.

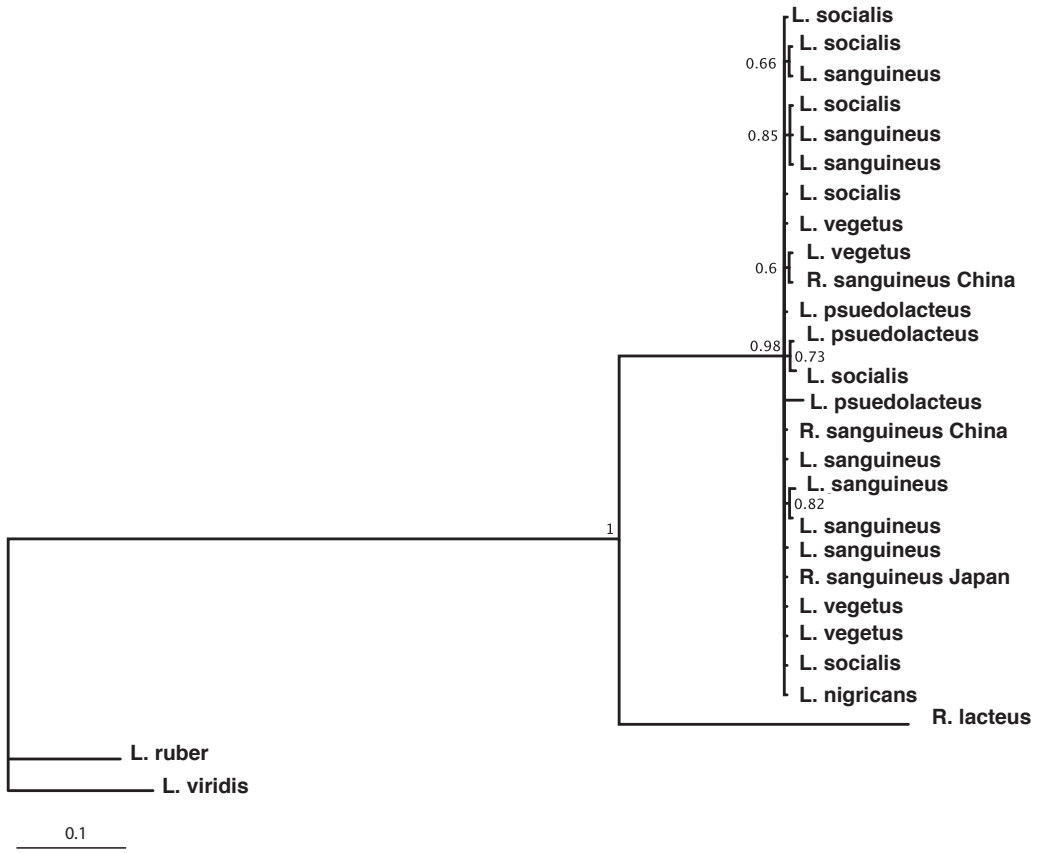

Figure 5. Bayesian Tree Based Delimitation Using *nad6* Sequences of Representatives of all "Geographic Species." *R. sanguineus* former designations are indicated as *L. socialis*, *L. psuedolacteus, L. sanguineus, L. vegetus* and *L. nigricans* and were originally defined on the basis of geographic location. These do not form distinct groups. The reference taxa are *L. ruber, L. viridis* and *L. lacteus* Boot strap values are noted. Numbers at nodes are posterior probabilities.

Tables

Table 1. Collection Sites and Former Designations. Former designation of species, population numbers, location, population names, number of individuals (N) and latitude and longitude for all collected samples of *R. sanguineus.*

Former Designation	Pop #	Location	Population	N	Lat	Long
Lineus socialis	**Atlantic North American shores**					
	ME8	Manset, ME		4	44.269956°	-68.312586°
	ME9	Burnt Island, ME	Penobscot Bay	2	44.000281°	-69.118486
	ME30	Phippsburg, ME	Popham Beach	52	43.749183°	-69.780103°
	NH31	Hampton, NH	Rye Beach	3	42.964756°	-70.769772°
	MA11	Nahant, MA		14	42.428222°	-70.916697°
	RI1	Newport, RI	Bretton Point State Park	64	41.451036°	-71.356469°
	NJ10	New Jersey		3	39.989989°	-74.060819°
	NY29	Long Island, NY	Montauk	2	41.046992°	-71.958778°
	SC2	Pawley's Island, SC	Pawley's Island Pritchard St groin	30	33.246400°	-79.079019°
	SC3	Pawley's Island, SC	Pawley's Island South End groin	30	33.240006°	-79.081703°
	SG#1 SC2	Pawley's Island, SC	Pawley's Island Pritchard St groin	10	33.246400°	-79.079020°
	OG#1 SC2	Pawley's Island, SC	Pawley's Island "old" groin	7	33.247239°	-79.078381°
	SC12	South Carolina		4		
	NC5	Wilmington, NC	Wrightsville Beach	1	34.188656°	-77.812961°
	FL28	Florida	Link Port groin	3	27.535081°	-80.347344°
Lineus vegetus	**Pacific North American shores**					
	WA4	San Juan Island, WA	Friday Harbor	8	48.543108°	-123.016000°
	CA23	Corona del Mar, CA		1	33.592892°	-117.876039°
	CA24	San Diego, CA	Bird Rock	1	32.814499°	-117.273376°
	CA25	Tomales, CA	Tomales Bay	6	38.166092°	-122.910894°
	CA26	Pacific Grove, CA	Hopkins Marine Lab	5	36.620308°	-121.904406°
	CA27	Palo Alto, CA	Byxee Park	4	37.456931°	-122.094972°
Lineus sanguineus	**Shores of European seas**					
	SP17	Bizkaia, Spain	Arminza	4	43.420381°	-2.953017°
	UK15	Anglesey, UK	Isle of Anglesey	1	53.316600°	-4.630875°
	UK16	Wales, UK	Llandudno	13	53.329142°	-3.827806°
Lineus psuedolacteus	**Shores of the English channel**					
	UK13	Great Britain, UK	Salcombe	17	50.314383°	-4.073019°
Lineus nigricans	**Mediterranean coast**					
	FR22	France	Seawater table	6	43.599382°	4.135203°
Unknown	JA19	Japan	Seto Aquarium	11	33.692697°	135.338197°
	JA20	Japan	Akkeshi Marine Lab beach	7	44.023258°	144.840947°
	CH14	China		11	36.003339°	120.309144°
Total				324		

Table 2. *Nad6* AMOVA Values. Mitochondrial *nad6* sequence data were obtained and AMOVA values calculated using GeneticStudio. Below diagonal: p-values. Above diagonal: pairwise Φ_{st} values. Moderate levels of differentiation are bolded. All other pairs of populations show significant differentiation. North (n=75) contains Maine (n=58), Massachusetts (n=14) and New Hampshire (n=3); Rhode Island (n=69) contains Rhode Island (n=64), New Jersey (n=3) and New York (n=2); South (n=85) contains South Carolina (n=81), North Carolina (n=1) and Florida (n=3); West (n=25) contains California (n=17) and Washington (n=8); Europe (n=41) contains United Kingdom (31), France (n=6) and Spain (n=4); Japan (n=18) contains Japan populations; China region (n=11) which contains the China population.

	North	Rhode Island	South	West	Europe	Japan	China
North	-	0.5642	0.6716	0.7380	0.4689	0.8002	0.7246
Rhode Island	0.0010	-	**0.1062**	**0.2909**	**0.0985**	0.4426	**0.2856**
South	0.0010	0.0010	-	0.5623	**0.2146**	0.7350	0.5386
West	0.0010	0.0010	0.0010	-	**0.1532**	**0.1435**	0.3643
Europe	0.0010	0.0010	0.0010	0.0010	-	**0.2532**	**0.1998**
Japan	0.0010	0.0010	0.0010	0.0010	0.0010	-	0.7619
China	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	-

Table 3. ISSR AMOVA Values. Nuclear inter-simple repeats (ISSR) data were obtained and AMOVA values calculated using GeneticStudio. Below diagonal: p-values. Above diagonal: pairwise Φ_{st} values. Moderate levels of differentiation are bolded. All other pairs of populations show significant differentiation. North (n=75) contains Maine (n=58), Massachusetts (n=14) and New Hampshire (n=3); Rhode Island (n=67) contains Rhode Island (n=62), New Jersey (n=3) and New York (n=2); South (n=85) contains South Carolina (n=81), North Carolina (n=1) and Florida (n=3); West (n=23) contains California (n=15) and Washington (n=8); Europe (n=37) contains United Kingdom (n=31) and France (n=6); Japan (n=17) contains individuals from Japan populations.

	North	Rhode Island	South	West	Europe	Japan
North	-	0.5103	0.7224	0.6034	0.4338	0.7612
Rhode Island	0.0010	-	**0.2096**	**0.2577**	**0.1321**	0.3469
South	0.0010	0.0010	-	0.5176	0.4243	0.6771
West	0.0010	0.0010	0.0010	-	**0.2244**	**0.1995**
Europe	0.0010	0.0010	0.0010	0.0010	-	0.3574
Japan	0.0010	0.0010	0.0010	0.0010	0.0010	-

Table 4. ISSR Synopsis. ISSR primer number, primer sequence, size range of bands, total number of bands scored for each primer and total number of bands scored for all primers.

Primer	Primer Sequence	Size range of bands (kb)	Total no. of bands scored
810	$(GA)_8T$	1900-3100	5
826	$(AC)_8C$	700-3000	10
842	$(GA)_8YG$	325-3200	13
Total			**28**

Table 5. ISSR Descriptive Statistics. N is the number of individuals in each region. The average number of bands, number of bands scored, number polymorphic bands and percent polymorphic for primers 810, 826, and 842 for each region as well as the total for all regions combined. The average number of bands overall for all primers as well as average number of bands per primer and the percent polymorphic for all regions and the total for the combined regions. Each population was combined based on AMOVA results and small sample size.

	North	**Rhode Island**	**South**	**West**	**Europe**	**Japan**	**Total**
N	75	67	85	23	37	17	304
810							
Avg # of Bands	1.87	2.27	2.69	1.35	1.46	1.65	2.09
No. of Bands Scored	3	4	4	3	4	2	5
No. Polymorphic Bands	3	4	4	3	4	1	5
% Polymorphic	100%	100%	100%	100%	100%	50%	100%
826							
Avg # of Bands	4.25	6.61	6.76	6.17	5.16	6.35	5.85
No. of Bands Scored	9	9	10	10	10	7	10
No. Polymorphic Bands	9	8	9	7	10	1	10
% Polymorphic	100%	89%	90%	70%	100%	14%	100%
842							
Avg # of Bands	6.73	6.90	7.05	6.65	7.11	7.35	6.93
No. of Bands Scored	11	11	10	11	13	10	13
No. Polymorphic Bands	10	11	8	9	12	5	13
% Polymorphic	91%	100%	80%	82%	92%	50%	100%
All Primers							
Avg # Bands Overall	12.85	15.78	16.51	14.17	13.73	15.35	14.87
Avg # of Bands/ Primer	4.28	5.26	5.5	4.72	4.58	5.12	4.96
No. of Bands Scored	23	24	24	24	27	19	28
No. Polymorphic Bands	22	23	21	19	26	7	28
% Polymorphic Bands	96%	96%	88%	79%	96%	37%	100%

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